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Nodular bacterial endophyte diversity associated with native *Acacia* spp. in desert region of Algeria

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Five species of *Acacia (Acacia ehrenbergiana* Hayne, *A. nilotica* (L.) Delile, *A. seyal* Delile, *A. tortilis* (Forssk.) Hayne and *A. laeta* Delile) indigenous to Tamanrasset (Algeria) were investigated for their nodulation status and nodular endophytic diversity. *A. ehrenbergiana* showed the highest nodulation ability across the different sites in this region, indicating the widespread occurrence of compatible rhizobia in the soils. Altogether 81 strains were purified. Among this endophytic strain collection, only four bacterial endophytes nodulated their respective host plants. On the basis of partial 16S rDNA sequencing, they were affiliated to *Ensifer* sp., *Ensifer teranga*, *Mesorhizobium* sp. and *Rhizobium* sp. Among the 79 non- symbiotic endophytes, 24 representative strains on the basis of PCR-RFLP profile obtained with MSPI enzyme digestion were characterized. They belonged to nine genera, namely: *Paenibacillus, Ochrobactrum, Stenotrophomonas, Pseudomonas, Microbacterium, Rhizobium, Agrobacterium, Brevibacillus* and *Advenella.* The isolated nodular endophytes in this study revealed a strong tolerance profile to salinity and high temperature. Principal component analysis confirmed that no correlation was found between bacterial tolerance to a maximum temperature of growth and soil depth of sampling. This tolerance profile was distributed over the three levels of soil depth sampling: 20, 40 and 60 cm. On the other hand, there was no relationship between *in vitro* tolerances of rhizobial strains to NaCl and high temperature and corresponding edaphoclimatic characteristics of the sampling sites. This study is a contribution to nodular bacterial diversity knowledge of desert African *Acacia* species growing in preserved ecosystems.

Key words: *Acacia* spp., bacterial endophytic diversity, desert, nodules, salinity tolerance, temperature tolerance.

INTRODUCTION

Endophytes can be defined as non-phytopathogenic organisms, which colonize plant tissues at least part of their lifetime (Wilson, 1995). They could accede to all vegetal compartments passively or actively (Hardoim et

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al., 2008). This is true for legumes, which are characterized by their ability to establish symbiotic relationships with Bacteria Nodulating Legumes (BNL). This plant-microorganism interaction gives rise to a neo-organ: the nodule where atmospheric nitrogen fixation takes place. It may host in addition to "nodulating endophytes" (BNL) a great variety of endophytic bacteria, including some rhizobial non-nodulating strains together with non rhizobial strains. Scientific attention focused on diversity of nodular endophytic bacteria, especially for cultivated species of legumes (Sturz et al., 1997; Bai et al., 2002; Li et al., 2008; Stajković et al., 2009; Ibanez et al., 2009). These efforts converged on improving crop yield both in quality and quantity. In contrast, little attention has been paid to the root-nodule bacterial endophyte of truly wild legumes; those whose ecology is not directly affected by human activities (Muresu et al., 2008) especially the trees. Endophytes have become in the last decades of a great interest due to their biotechnological applications. Their beneficial effects had been reviewed intensively as they could act as Plant Growth Promoters (PGP), enhance plant resistance to stress and disease and are producers of active secondary metabolites (Compant et al., 2010).

Tamanrasset is a vast desert region with an annual rainfall of 48 mm and mean temperature that ranges from -1 to 48°C following Algerian National Organization of Meteorology –ONM (2006). It is located in the South-east of Algeria. It harbors an interesting flora and is the only Algerian region where five indigenous species of *Acacia* are localized (Sahki et al., 2004; Ozenda, 1983): *Acacia ehrenbergiana* Hayne, *A. nilotica* (L.) Delile, *A. seyal* Delile, *A. tortilis* (Forssk.) Hayne and *A. laeta* Delile. These rustic leguminous trees survive under extremely harsh conditions of drought in dry oueds (dry rivers), are widely used for animal and human medicines preparation, and are source of fuel and animal feeding. In their natural distribution area, most of the African *Acacia* spp. were associated with *Mesorhizobium* (de Lajudie et al., 1998; Haukka et al., 1998; McInroy et al., 1999; Ba et al., 2002; Odee et al., 2002), *Ensifer* (syn. *Sinorhizobium*) (de Lajudie et al., 1994; Haukka et al., 1998; Khbaya et al., 1998; Ba et al., 2002) and, to a lesser extent, *Rhizobium* (McInroy et al., 1999; Nick et al., 1999) and *Bradyrhizobium* (Dupuy et al.,1994; McInroy et al., 1999; Odee et al., 2002). Some studies revealed leguminous tree non-symbiotic nodular endophytes as *Conzattia multixora* grown in Mexico (Wang et al., 2006), woody and shrub legumes growing in Ethiopia: *Crotalaria* spp., *Indigofera* spp., and *Erythrin abrucei* (Aserse et al., 2013). Many scientists working on African *Acacia* species symbionts did encounter bacteria non-nodulating endophytes; for example: *Ensifer meliloti, Rizobium tropici* and *Agrobacterium* –like strains associated with *A. tortilis* in Tunisia (Ben Romdhane et al., 2005); *Agrobacterium* associated with *Acacia gummifera* in Morroco (Khbaya et al., 1998) and *A. cyclops* and *A. mollissima* in Senegal (de Lajudie et al., 1999). Only one study (Hoque et al., 2011) did reveal non-symbiotic endophytic bacteria diversity associated with two species of *Acacia*, namely *Acacia salicina* and *Acacia stenophylla* in arid and semi-arid regions of Australia. The aim of this work is to reveal nodulation status of *Acacia* species indigenous to Tamanrasset and the diversity of endophytic bacteria isolated from their nodule; it also aims to establish a possible relationship between bacterial affiliation/plant host and bacterial tolerance profile/ depths of soil sampling (20, 40 and 60 cm surrounding native trees).

MATERIALS AND METHODS

Soil sampling and site description

Soil samplings were collected from three depths (20, 40 and 60 cm) in a maximum diameter of 1meter around the trunks of vigorous trees. Each soil sample was kept in sealed bag for further use. Prospect soils were neutral to slightly alkaline and non-saline except Oued Idekel with CW of 0.637 which is considered as slightly saline according to Durand scale (1983) (Table 1). All soils showed rocky, sandy and loamy texture, but Djnan Biga which was loamy, clayey and sandy. *Acacia* species were encountered in or on the sides of dried rivers (oueds). Tree species were not distributed homogeneously in the eight prospected sites. There is generally a numerical predominance of *A. ehrenbergiana* and *A. tortilis* when present in the studied sites.

Nodules sampling

Sampling was conducted in November (Rainy season is generally situated between June and September). Tree root nodules were prospected at the three depths cited above. No nodules were found *in natura*. Seeds were harvested by the trees of every sampling site when they were present; for *Acacia laeta*, they were supplied gracefully by Forestry Direction of Tamanrasset. Trapping assay was performed using the collected soil with seeds of associated *Acacia* species: seeds of native species *Acacia nilotica*, *A. ehrenbergiana*, *A. seyal*, *A. tortilis*, and *A. laeta* were scarified and surface sterilized by treating with concentrated (95%) H_2SO_4 for 120, 30, 30, 30, and 20 min, respectively. They were then rinsed thoroughly with sterile distilled water and germinated on 0.8% (w/v) water agar at 28°C for two to five days. The germinated seeds were then transferred in aseptic conditions into Gibson tubes (Gibson, 1980) containing sterile Jensen nitrogen-free medium (Vincent, 1970). Plants were grown in an incubation chamber as described by Diouf et al. (2007). After 1 week of growth, 1 mL of stirred soil suspension was added to each tube. Soil suspension was obtained from 10 g of each soil sample added to 90 mL of sterile buffered saline, pH 7 (NaCl 0.15 M, KH_2PO_4 0.002 M, Na_2HPO_4 0.004 M) and shaken for 1 h. Four replicates were tested for each soil sample. Un-inoculated plants served as controls. 208 tubes were prepared. Plants were observed for nodule formation 6 weeks after germination, and fresh nodules were collected and conserved under desiccated condition in tubes containing $CaCl_2$ crystals.

Bacterial strains isolation and culture conditions

The root nodules were washed by immersion in 70% (v/v) ethanol for 30 s, rinsed with sterile distilled water, then rehydrated for 1 h.

Table 1. Sampled soil characterization and localization and *Acacia* spp. nodulation status.

Sampling sites localization in Tamanrasset	*Acacia* species encountered	Number of nodulated plants/4Gibson tubes inoculated by 10^{-1} of soil suspension	Depth of soil sampling cm	Soil conductivity (mS/cm^2)	Soil pH
Djnen Biga 22°33.683 N 005°23.754 E ALT 3735FT	*A. ehrenbergiana*	3	20	ND	ND
Oued In Deladg 22°56.004 N 005°52.851 E ALT 4554FT	*A. albida*	1	20	0.105	7.70
		2	40		
		2	60		
	A. ehrenbergiana	4	20		
		2	40		
		3	60		
	A. nilotica	0	20		
		2	40		
		2	60		
Oued Tassena 35°35.240 N 000°48.527O ALT 358FT	*A. albida*	0	20	0.078	8.17
		0	40		
		0	60		
	A. tortilis (predominant)	2	20		
		4	40		
		4	60		
	A. leata	3	20		
		3	40		
		2	60		
	A. ehrenbergiana	2	20		
		4	40		
		3	60		
Oued Tamezzegine 22°35.218 N 005°23.815 E ALT 3740FT	*A. ehrenbergiana*	3	20	0.149	7.8
		4	40		
		4	60		
	A. nilotica	2	20		
		0	40		
		1	60		
	A. tortilis	3	20		
		4	40		
		3	60		
Oued Idekel 22°33.783 N 005°24.020E ALT3732 FT	*A. nilotica*	0	20	0.637*	7.8
		0	40		
		1	60		
Oued Taghemout 22°58.640 N 005°40.892 E ALT 5461FT	*A. seyal* (predominant)	0	20	0.148	7.7
		2	40		
		0	60		
	A. seyal	0	20		
		0	40		
		1	60		
	A. tortilis	0	20		
		0	40		
		0	60		

Table 1. Contd.

Oued In Tounin (Tasekra) 22°56.004 N 005°52.851 E ALT 4554FT	*A. tortilis* (predominant) *A. ehrenbergiana*	0 1 1 2 3 3	20 40 60 20 40 60	0.326	7.69
Oued Tan-Assennane 22°44.543 N 005°38.291 E ALT 4112FT	*A. leata*	0 2 1	20 40 60	0.082	7.04

*****Soil slightly saline.

They were individually surface-sterilized by immersion in 35% H_2O_2 for 15 s to 2 min according to their size, and then rinsed in successive baths of sterile distilled water. After crushing, the nodule homogenates were spread onto yeast extract-mannitol agar (YMA) plates (Vincent, 1970). One to three isolated colonies per nodule homogenate were obtained from successive streaking on YMA plates. Their purity and membrane nature were checked by phase-contrast microscopy and Gram coloration respectively. Colony shape and color were determined using a magnifying glass. Pure cultures were then cryogenically preserved (-80°C) in yeast extract-mannitol medium (YEM) adjusted to 30% (v/v) glycerol.

Tolerance of bacterial isolates to salinity and temperature

All tests were carried out in triplicate. Inoculation isolates were grown in YEM liquid medium to log phase (corresponding to approximately 10^9 colony-forming units mL^{-1}). In test plates, inoculation was performed with 10 µl of these cultures. The results were scored after 7-day incubation. Positive results were reported when bacterial colonies were observed under a binocular microscope at 950 magnification. Tolerance to salinity was tested on YEM agar plates containing NaCl concentrations of 170, 340, 510, 680, 860, and 1034 mM incubated at 28°C. For temperature tolerance, YMA plates were inoculated and incubated at four different temperatures: 35, 40, 45, and 50°C.

Nodulation test

The nodulation ability of the obtained bacterial isolates was checked by inoculating seedlings of the corresponding *Acacia* species grown in hydroponic conditions. Tubes were filled with Broughton and Dilworth N-free nutrient solution (Broughton and Dilworth, 1971); a tab paper for plant support was introduced, then the tubes were sealed with aluminum folder and a rubber band and were sterilized. After cooling, central hole was dug for seedling introduction. One week after germination, three plants per Acacia species were inoculated with approximately 2 mL of bacterial suspension at 10^9 cells mL^{-1} of each isolate. Uninoculated controls were included. Plants were transferred to a greenhouse and watered with a sterilized Broughton and Dilworth (1971) N-free nutrient solution (Broughton and Dilworth), when it was necessary. Five weeks after inoculation, plants were harvested to estimate the infectivity and effectivity of bacterial isolates based on the presence of nodules, nodule color, visual observation of plant vigor, and foliage color. The bacterial isolates were recorded as nodulating or non-nodulating.

16S PCR-RFLP

Selected and isolated colonies were homogenized into sterile 1.5-mL centrifuge tubes with 50 µl of sterile Milli-Q water; suspensions were centrifuged at 6000 g for 5 min and washed twice. Pellets were re-suspended and then stored at 20°C until PCR use. The universal eubacterial 16SrRNA gene primers FGPS 6 (5′-GGA GAG TTA GATCTT GGC TCA G-3′) and FGPS 1509 (5′-AAG GAGGGG ATC CAG CCG CA-3′) (Normand et al., 1992) were used, and PCR was performed as follows: An initial cycle of denaturation at 94°C for 5 min, 36 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 2 min, and a final extension at 72°C for 7 min. The PCR products were checked by 0.8% (w/v) agarose gel electrophoresis in Tris/acetate/ EDTA (TAE) buffer (40 mMTris-acetate, 1 mM EDTA, pH 8.3). Bands were excised and DNA was purified using a QIAquick gel extraction kit (Qiagen, Courtaboeuf, France). The final 16S ADN product was digested by restriction enzyme MPS I (Promega) as follows: 8 µ of PCR product, 2 µL buffer 10X, restriction enzyme (10U/µL) 0.5 µL and qsp 20 µL PCR water. The restriction digests were incubated during 1 h 30 min at 37°C and checked on agarose-gel (3%) electrophoresis to ensure they had gone to completion in TBE buffer (89 mmol Tris base, 89 mmol boric acid, 2 mmol EDTA, pH 8.0). The gels were run at 40V:15 min then at 80V: 1 h 45 min. Gels were stained by addition of ethydium bromide (1mg mL^{-1}), and photographed by RAISER camera (Germany) with Perfect Image software, V-5.3 (Clara vision). The gel profiles of RFLP pattern were done with phoretix 1D software (Totallab, 2013). The patterns were converted to matrix of zero and one depending on the presence or absence of bands for different strains. Dice's similarity coefficients were then calculated between pairs of bands. Strains were grouped by using the unweighted pair group method with arithmetic averages (UPGMA), dendrogram construction utility (DendroUPGMA) at S. Garcia-Vallve, Biochemistry and Biotechnology Department, Rovira i Virgili University, Tarragona, Spain [http://genomes.urv.cat/UPGMA/] and tree dyn application (Dereeper et al., 2010).

Partial 16S rRNA gene sequencing

The final 16S ADN product (following the methodology cited previously) was sequenced using primers FGPS6, FGPS1509, and 16S-1080r (5′-GGG ACT TAA CCC AAC ATC T-3′) (Sy et al., 2001). Sequencing reactions were analyzed on an Applied Biosystems model 310 DNA automated sequencer using a Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer Applied Biosystems). Sequences were corrected using CHROMASPRO 5

(Technelysium Pty Ltd., Tewantin, Australia) before alignment and analysis of the 16S rRNA gene sequences using CLUSTAL X software (Thompson et al., 1997). Phylogenetic analysis was inferred using the neighbor-joining method (Saitou and Nei, 1987) calculated by the Kimura method (Kimura, 1980). The 16S rRNA gene sequences of the type strains of the various genera used in this study were retrieved from the GenBank/EMBL database and used for cladistic analysis. A bootstrap analysis using 1000 replications was performed. The 16S rRNA gene sequences of selected Acacia non-nodulating strains were deposited in the GenBank database under accession numbers (Table 2) and were included in the phylogenetic tree.

Statistical analyses

Principal component analysis (PCA) was performed to examine the relationships between depth of sampling and *in vitro* bacterial tolerance to salinity and maximum temperature of growth, and a Factorial correspondence analysis (FCA) was performed to visualize the relationships between rhizobial taxa, as defined from the partial 16S rRNA gene-based phylogeny, and Acacia host species. Computations and graphical display for both methodologies were made using the XLSTATTM software package (version 2010.5.04, Addinsoft, Paris, France, http://www.xlstat.com)

RESULTS AND DISCUSSION

Nodulation status of Tamanrasset native *Acacia* species

Tamanrasset is considered as a desert region with an annual rainfall less than 100 mm. Sampling was conducted in November, two months after the rainy season to optimize the chances to find nodules, but none was encountered *in natura*. Host trapping assay showed the highest number of nodulated plants inoculated with soil suspension obtained from 40 and 60 cm instead of 20 cm of sampling depth. This could be due to relative soil humidity which allows rhizobial surviving; in stressful environments compatible rhizobial populations may be transiently insufficient or non-existent in the surface soil (Wolde-meskel et al., 2004).

There was no nodulation on root of *A. albida* with Oued Tassena soil suspension contrary to that of Oued in Daladg. The same goes for *A. laeta* which was poorly nodulated in association with Oued Tan-Assennane soil compared to those of Oued Tassena. Whereas, *A. nilotica* was poorly nodulated whatever the soil origin. This could be due to the low level of compatible rhizobial population. The highest nodulation pattern was observed on roots of *A. ehrenbergiana* followed by *A. tortilis.* Both species were dominant numerically when they were present at the prospected sites, so we could assume that rhizobial population correlates to tree's density as reported by Thrall et al. (2007).

The obtained nodules had a determinate and indeterminate shape whatever host species and prospected site; while observed Acacia's nodules in literature were indeterminate and woody (Sprent, 1989).

Nodular endophytic bacteria phentotypic and symbiotic characterization

81 isolates were obtained from nodules of the five *Acacia* spp. All purified strains showed fast growth (2 to 3 days). The majority of colonies was translucent or white watery; some were pointy in form and others were "marbled" (milky curdled type). All were gram negative coccobacillus with the occurrence of some rods.

Only four endophytic bacteria were true symbiont by their nodulation ability of host trap species (Table 2). This paucity of nodulation for endophytic nodular bacteria had been reported for true wild legumes by Muresu et al. (2008). These authors had given some hypotheses to explain this phenomenon: sterilization techniques did not allow rhizobial recovery; moreover salinity or acidity of isolation culture media could interfere with surviving of rhizobia. Finally the predominant endophytic non rhizobial bacteria may produce antagonistic compound affecting symbiont growth.

There were no marked difference between uninoculated plants and inoculated ones, so we could not suggest a plant growth promotion effect of these non nodulating endphytes.

Nodular endophytic bacteria genetic diversity

The blast results from nearly full-length 16S rRNA gene sequences (> 1300 nt) for symbiotic nodulating bacteria classified them as *Ensifer teranga*, *Ensifer* sp., *Mesorhizobium* sp. and *Rhizobium* sp. (Table 2). These results are in agreement with studies on African symbiont diversity (de Lajudie et al., 1994, 1998; Nick et al., 1999). Among the 79 non nodulating endophytes, 24 representative strains were genetically characterized. Bacteria were selected on the basis of their macroscopic aspects and PCR-RFLP profile after digestion with MSPI enzyme. In our study, it is highly discriminating. This discrimination power has also been reported by Fall et al. (2008). Selected strains belonged to nine genera (Table 2) namely: *Paenibacillus, Ochrobactrum, Stenotrophomonas, Pseudomonas, Microbacterium, Rhizobium, Agrobacterium, Brevibacillus, Advenella*. On the basis of phylogenetic study, five major groups were distinguished (Figure 1). The first group included nodulating reference strains and an *Agrobacterium* sub-group affiliated with *Agrobacterium tumefaciens* ; it included two strains blasted as *Rhizobium* sp. (N141a, L120T) and *Agrobacterium tumefaciens* (N70a) ; the second sub-group included *Ochrobactrum* genus: E85b close to *O. intermedium* ; and L30b, S180b, E46b close to *O. anthropi*. The second group was constituted by *Pseudomonas aeruginosa* sub-cluster close to N141B, E136a; an *Advenella kashmirensis* branch associated with T21b and *Stenotrophomonas maltophila* close to N97b3, E136c, N97B. For the two other clusters, the first is affiliated to *Microbacterium oxydans* including N142b2,

Table 2. Molecular and tolerance profile characterization of *Acacia* species nodular endophytes.

Strains (Accession number)	Closest partial 16S rRNA Gene sequence *	Maximum strain temperature tolerance (°C)	Maximum strain salinity tolerance (mM)	Associated host plant	Site of sampling localization	Depth of sampling (cm)
Renodulating						
E60 (HQ836175)	*Ensifer teranga*	50	340	*A. ehrenbergiana*	Oued Tin Amezzegin	60
T82 (HQ836175)	*Ensifer* sp.	40	1034	*A. tortilis*	OuedTassena	60
A121 (HQ836160)	*Mesorhizobium* sp.	40	680	*F. albida*	Oued In Daladg	60
N145 (HQ836162)	*Rhizobium* sp.	40	680	*A. nilotica*	Oued In Daladg	60
Non nodulating						
E85b (KM894187)	*Ochrobactrum intermedium*	40	1034	*A. ehrenbergiana*	Djnen Biga 22°33.683 N 005°23.754 E ALT 3735FT	20
E136c (KM894178)	*Stenotrophomonasmaltophilia*	50	1034	*A. ehrenbergiana*		60
E136a (KM894176)	*Pseudomonas aeruginosa*	45	1034	*A. ehrenbergiana*		60
N142B (KM894185)	*Microbacterium oxydans*	40	1034	*A. nilotica*	Oued In Deladg 22°56.004 N	40
N142b2 (KM894196)	*Microbacterium oxydans*	40	1034	*A. nilotica*	005°52.851 E ALT 4554FT	40
N141B (KM894177)	*Pseudomonas aeruginosa*	45	1034	*A. nilotica*		40
N141a (KM894198)	*Rhizobium* sp.	50	1034	*A. nilotica*		40
L120T (KM894194)	*Rhizobium* sp.	40	680	*A. laeta*		40
E46b (KM894190)	*Ochrobactrum anthropi*	40	1034	*A. ehrenbergiana*		60
L30b (KM894179)	*Ochrobactrum anthropi*	40	1034	*A. laeta*	Oued Tassena 35°35.240 N	40
L29b (KM894183)	*Peanibacillus*sp.	50	510	*A. laeta*	000°48.527O ALT 358FT	40
T21b (KM894192)	*Advenella kashmirensis*	40	1034	*A. tortilis*		60
T20C (KM894195)	*Paenibacillus humicus*	40	510	*A. tortilis*		40
N70a (KM894180)	*Agrobacterium tumefaciens*	40	1034	*A. nilotica*	Oued Tamezzegine 22°35.218 N 005°23.815 E ALT 3740FT	60
T76c (KM894181)	*Paenibacillus glycanilyticus*	40	510	*A. tortilis*		20
N97b3 (KM894189)	*Stenotrophomonasmaltophilia*	40	1034	*A. nilotica*	Oued Idekel 22°33.783 N 005°24.020EALT3732 FT	60
N97B (KM894184)	*Stenotrophomonasmaltophilia*	45	1034	*A. nilotica*		60
S178a (KM894182)	*Paenibacillus glycanilyticus*	50	1034	*A. seyal*	Oued Taghemout	40
S186a (KM894193)	*Paenibacillus humicus*	50	1034	*A. seyal*	22°58.640 N 005°40.892 E	20
S186B (KM894191)	*Brevibacillus nitrificans*	50	1034	*A. seyal*	ALT 5461FT	20

Table 2. Contd.

S180b (KM894186)	*Ochrobactrum anthropi*	45	1034	*A. seyal*		40
T160b (KM894199)	*Pseudomonas* sp.	45	680	*A. tortilis*	Oued In Tounin (Tasekra)	60
T159b (KM894197)	*Peanibacillus* sp.	40	1034	*A. tortilis*	22°56.004 N 005°52.851 E ALT 4554FT	60
L105b (KM894188)	*Peanibacillus* sp.	45	1034	*A. laeta*	Oued Tan-Assennane 22°44.543 N 005°38.291 E ALT 4F96FT	40

***** Similarity of 99%.

N142B and the second to *Brevibacillus nitrificans* associated with S186B. The last group included all the *Paenibacillus* genera: *Paenibacillus glycanilyticus* close to T76c, S178a and P*aenibacillus humicus* affiliated with S186a, T20C. We recorded a strong sub-group blasted as *Paenibacillus* sp. not affiliated to reference strains which included L105b, L29b and T159b.

N70a and N141a are at the same phylogenetic position; the first one is affiliated to *A. tumefaciens* while the second to *Rhizobium* sp. Moreover, in the phylogenetic tree (Figure 1), strain L120T has the same phylogenetic position as *A. tumefaciens* but is affiliated to *Rhizobium* sp.. These results highlighted the monophyletic status of *Rhizobium* and *Agrobacterium* (Young et al., 2001; Sawada et al., 2003; Farrand et al., 2003); even partial sequencing of 16S R DNA did not give an exact taxonomic position.

Generally, *Paenibacillus* is the second most abundant genera from nodule endophyte isolates (Velasquez et al., 2013). In our study, it was predominant. A study conducted by Hoque et al. (2011) on nodular endosymbiotic bacteria isolated from *Acacia stenophylla* and *Acacia salicina,* native species in Australia reported an unexpected diversity of about 19 genera. Eight of them are similar with our findings but *Advenella kashmirensis* was not reported as presenting a status of endophyte in literature. There are other studies on native legumes in arid and semi-arid regions in Tunisia (Benhizia et al., 2004, Zakhia et al., 2006); non-inoculated legumes in the Mediterranean basin (Muresu et al., 2008) and perennial *Lespedesa* in South Korea (Palaniappan et al., 2010) revealed the same or different affiliations as cited above, but always a remarkable diversity.

Tolerance profile to salinity and high temperatures of bacterial strains

Nearly 80% of characterized strains tolerated 1034 mM of salinity and five grew at 50°C (Table 2). A Principal Component Analysis (PCA) was used to explore variation of *in vitro* bacterial tolerance to salinity and a maximum temperature of growth according to depth of sampling. The three principal components accounted for 41.43, 36.06 and 22.5%, respectively. There was no relationship between these parameters. As clearly confirmed by PCA (Figure 1), no correlation was found between bacterial tolerance to a maximum temperature of bacterial growth and depth of sampling (r= -0.196); even a slight correlation was revealed between bacterial tolerance to salinity and depth of sampling with r= 0.201.

In a previous work, we found out (Boukhatem et al., 2012) that there were no relationship between *in vitro* tolerances of rhizobial strains to NaCl and high temperature and corresponding edaphoclimatic characteristics of the sampling sites. These findings were in agreement with this present study; maximum *in vitro* strain salinity tolerance was reported for bacterial endophytes originated from slightly saline soil (Oued Idekel) than those from non-saline sites (Table 1 and Figure 2). The isolated nodular endophytes in this study revealed a strong tolerance profile which made them to survive in soils suffering from harsh conditions. It was proven previously that salt-tolerant and temperature-resistant characteristics made the bacteria adapt to extreme environments (Tripathi et al., 2002; Tehei and Zaccai, 2005). This resistance profile was distributed over the three levels of soil sampling: 20, 40 and 60 cm (Table 2). Whilst, endophyte colonization may originate from other source other than rhizosphere, such as phyllosphere, anthosphere, or spermosphere (Hallmann et al., 1997). In this study, soil suspension trapping methodology was used. The main source of endophytes was soil and to a lesser degree endophytes were acquired by horizontal transfer (they were present into seeds). We suppose that in natural conditions, even bacteria presented a high tolerance profile. Environmental temperature could affect the fluctuation of bacterial endophytes (Mocali et al., 2003) and stressful

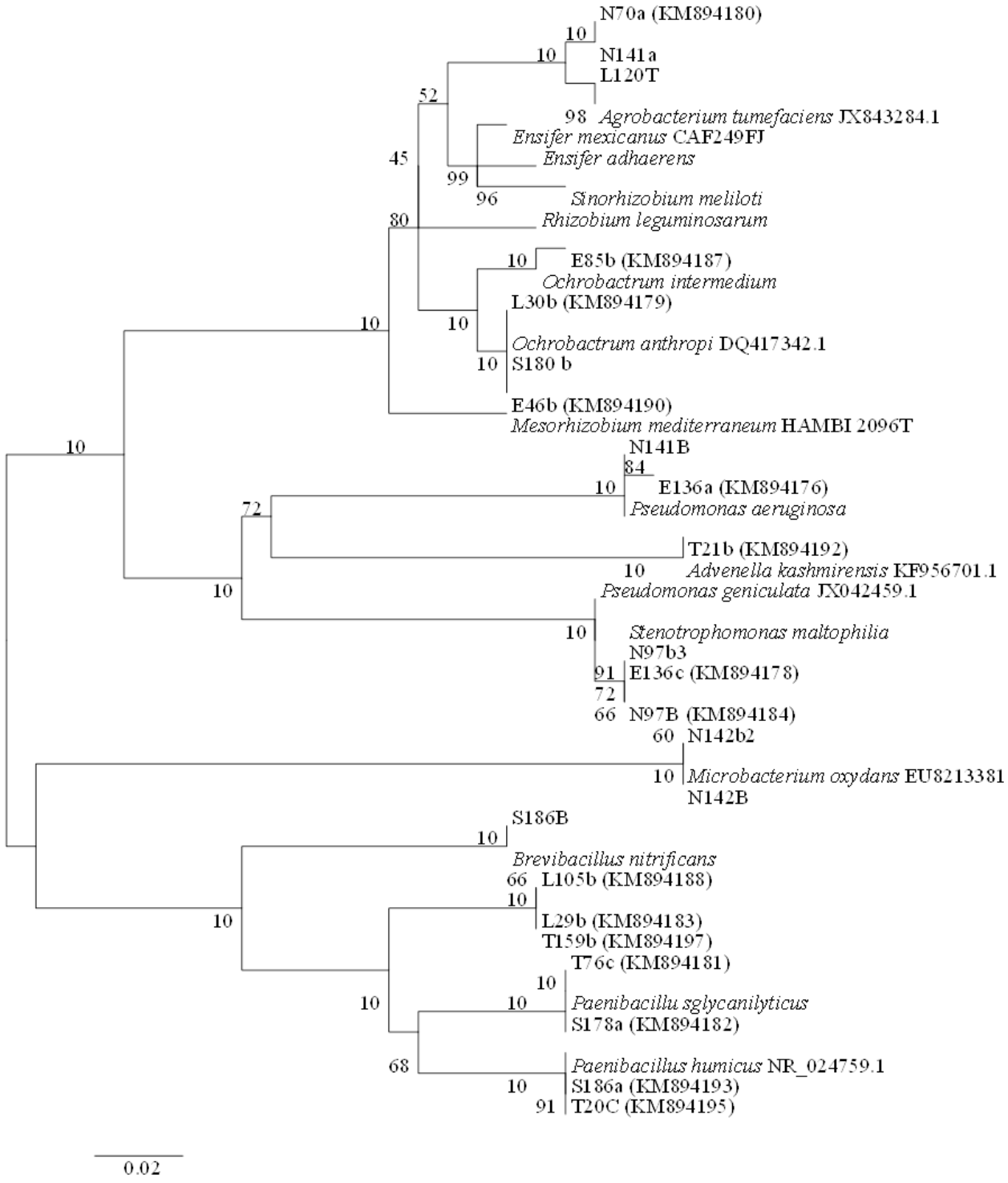

Figure 1. Phylogenetic tree based on nearly full-length 16S rRNA gene sequence (> 1300 nt) analysis of nodule endophytic bacteria of native *Acacia* sp. in Tamanrasset and reference strains (renodulating and endophytic). Neighbor-Joining method integrating Kimura 2 distance was used. Data are bootstrap values issued from 1000 repetitions. One strain listed in Table 1 was not included in the phylogenetic tree since its nucleotide length was <1300 nt.

conditions could drive the selection toward higher frequencies of infection as it has been proven for grasses associated with fungal endophytes (Jensen and Roulund, 2004). On the other hand, when legume plants are exposed to complex communities they selectively regulate access and accommodation of bacteria

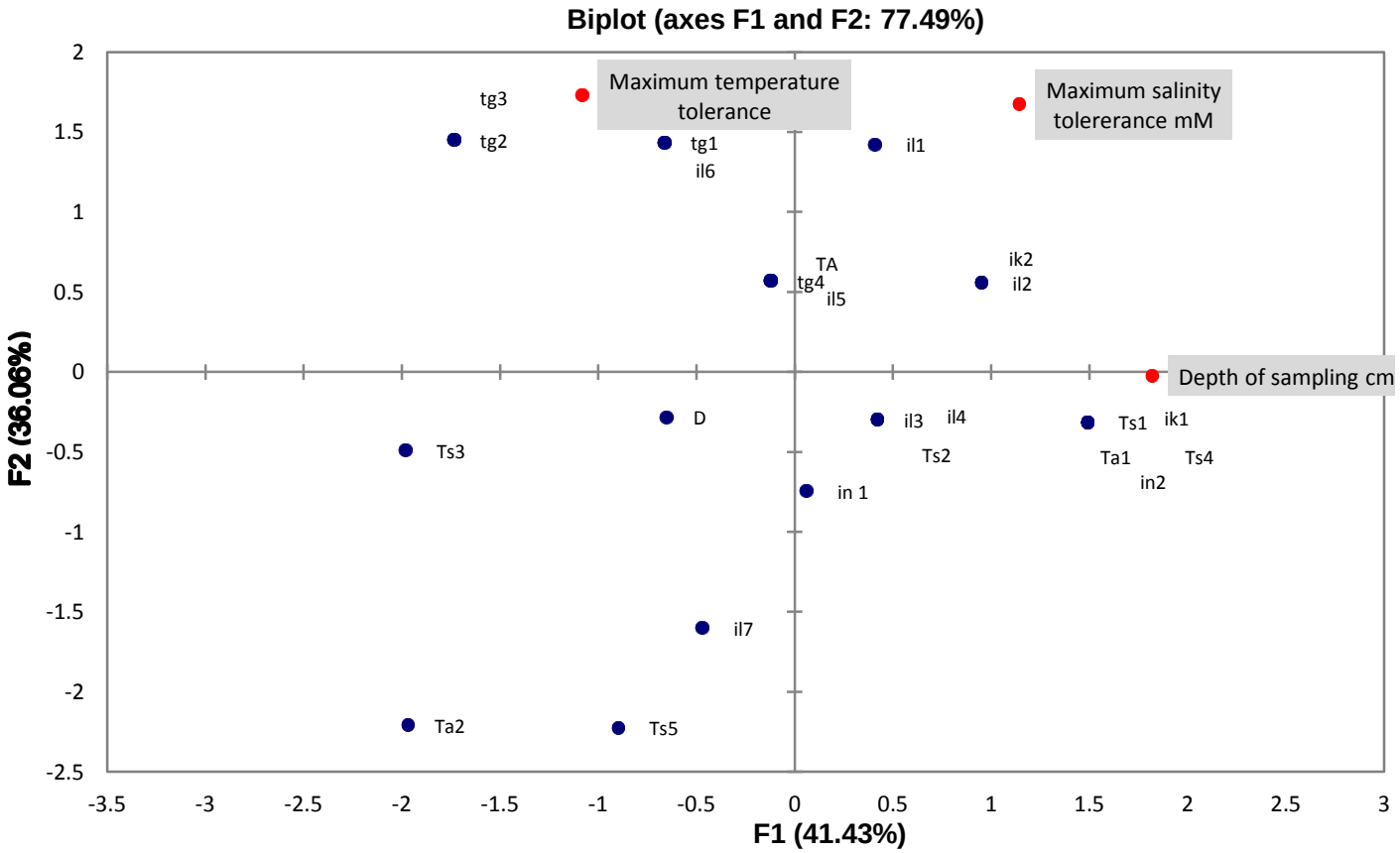

Figure 2. PCA representing the relationships between *in vitro* tolerance of nodular endophytic isolates to NaCl and high temperature and depth of sampling. Each dot corresponds to a single bacterial isolate from its site of sampling and is indicated by a symbol as follows: **D**: Djnen Biga; **il**: Oued In Deladj; **Ts**: Oued Tassena; **Ta**: Oued Tin Amezzejin; **ik**: Oued Idekel; **tg**: Oued Taghemout; **TA**: Oued Tan-Assennane, **in**: Oued In Tounin (Tasekra).

occupying the root nodule which is considered as a specialized environmental niche (Zgadzaj et al., 2015).

Plant host specificity

Acacia nilotica presented the largest panel of endophytic diversity due to the highest number of isolates (8). It did not harbor the dominant genera *Paenibacillus* or *Ochrobactrum*. Only *A. ehrenbergia* and *A. nilotica* share more than one genus (*Stenotrophomonas* and *Pseudomonas*) (Figure 3). The hypothesis that differentplant species can be colonized by a different spectrum of endophytic bacteria was given by McInroy and Kloepper (1995) in experiments conducted on sweet corn and cotton. Another study (Izumi et al., 2004) performed on coniferous and deciduous tree species by comparing the most abundant cultivable bacteria in the rhizosphere and root samples suggested that root's endophytic bacteria may be in residence through a process of selection or by active colonization rather than by passive diffusion from the rhizosphere. There are influencing factors prior colonization including plant-species-specific factors: root architecture, surface structure and root's exudates and non-plant factors like wounding or mycorhization. On the other hand after the root colonization, size of the intercellular space, nutrient composition within the apoplastic fluid and the plant's response to endophytic colonization are probably the main factors determining bacterial selectivity and thus the bacterial spectrum found inside the roots (Hallmann and Berg, 2006).

Conclusion

Tamanrasset hosts a great biodiversity of native *Acacia* spp. These trees are highly resistant to drought and survive under extremely harsh conditions. It is interesting to reveal bacterial diversity associated with their nodules, symbiotic and non-symbiotic, especially in this ecologically preserved and fragile ecosystem. The weak recovery of non nodulating bacteria gives rise to some questions about specific interactions between bacterial

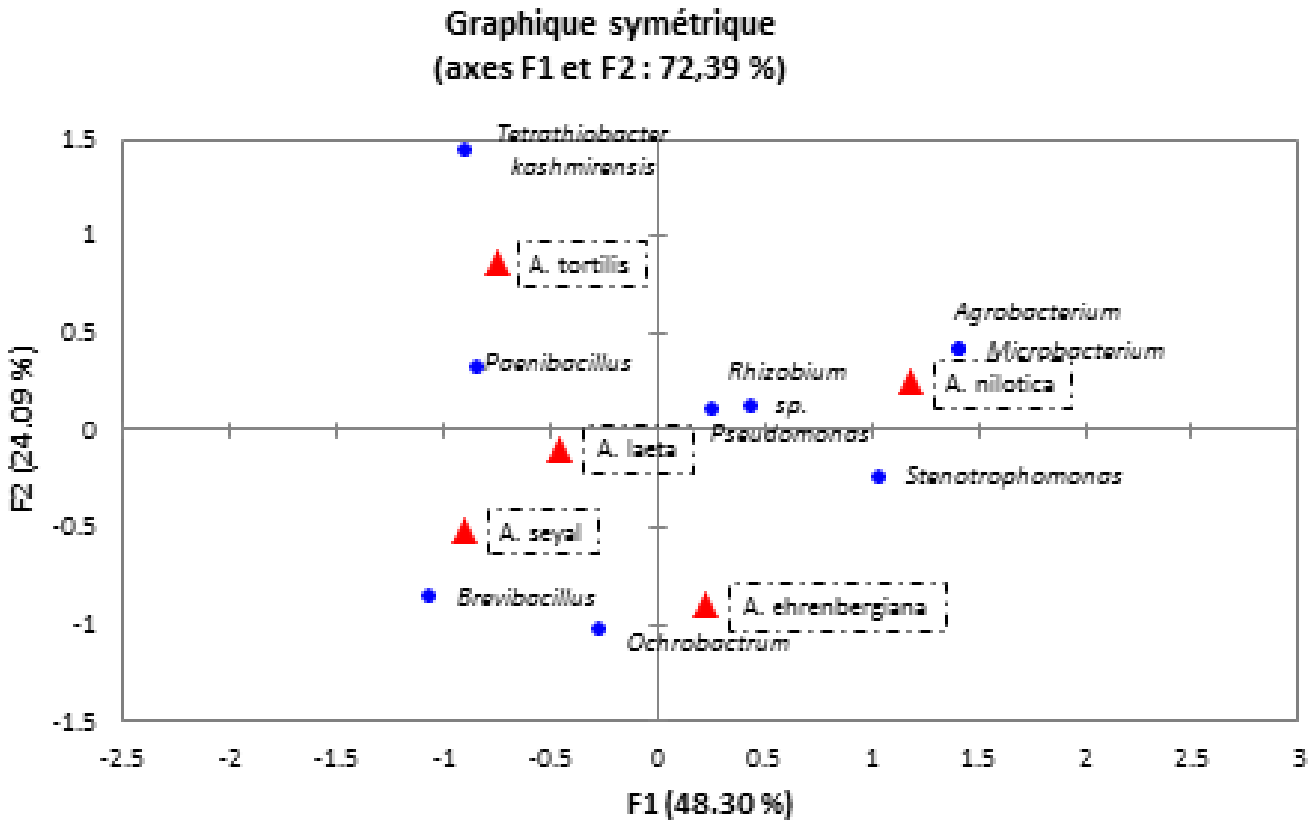

Figure 3. First factorial plane projection of FCA between endophytic bacterial taxa (circles), as defined from the 16S rRNA gene-based phylogeny, and five *Acacia* species (triangles).

endophytes and symbionts which initiate nodules. We wonder if endophytic population size or metabolite production inhibits rhizobial growth. Even these non-symbiotic endophytes did not show a PGP effects; other beneficial actions have to be investigated as their effect on seedling emergence, bio-control ability and production of metabolites with biotechnological uses.

Conflict of Interests

The authors have not declared any conflict of interests.

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